Naval Health Research Center Detachment
(Toxicology)

ACUTE TOXICITY ASSESSMENT OF BREAK-FREE CLP®: A WEAPONS CLEANING AND MAINTENANCE COMPOUND

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Abstract:

Break-Free CLP® ("Break-Free CLP") is a commercial cleaning, lubricating, and preserving compound used in both the military and civilian sectors for maintenance of small and large caliber weapons. Break-Free CLP is a complex mixture made up of polyalphaolefin oil (65%), synthetic oils, esters and other synthetic proprietary ingredients (27%), isoparaffinic hydrocarbons (5%), and dibasic ester (3%). Like many commercial mixtures, there is very little information available on the toxicity of Break-Free CLP. Studies were conducted to characterize the biological effects of single or repeat dermal application of Break-Free CLP to the clipped backs of CD-1 mice. Break-Free CLP was applied neat, 50 µL/application, 3 times/week (MWF) for up to 2 weeks. Foci of epithelial ulceration were observed in skin sections from 22% of Break-Free CLP animals in conjunction with markedly thickened epithelium, suggesting a robust process of epithelial regeneration was occurring in these animals. Skin histopathology of Break-Free CLP-treated animals closely matched those of mice treated repeatedly with 2% croton oil (CO) in acetone (dermal irritation positive control). Serum alkaline phosphatase (ALKP) concentrations were significantly ($p \leq 0.05$) lower for mice treated with Break-Free CLP, 2% croton oil, or 7,12-dimethylbenz(a)anthracene (DMBA) as compared with negative and vehicle control mice. Skin nitric oxide (NO) levels were not significantly elevated for mice treated with BreakFree CLP but were significantly elevated for mice treated with dermal irritation positive control compound DMBA. The cumulative skin changes in Break-Free CLP-treated animals support conducting a subchronic dermal application study. The observed decreases in serum ALKP levels suggest that future studies should include the liver and bone as possible target organs. Additionally, dermal penetration studies would provide key health risk assessment information for characterizing the potential health risks associated with chronic dermal exposure to Break-Free CLP.

Keywords: Break-Free CLP, polyalphaolefin, dermal effects, dermal toxicity, nitric oxide

Introduction

Cleaning of weapons is a very labor intensive, "hands-on" operation. Repeat dermal contact with weapons maintenance compounds during cleaning is highly probable, particularly when personnel protective equipment (PPE) is not used, readily accessible, or unavailable. Under combat conditions, cleaning and maintenance operations may be performed repeatedly on a daily basis for possibly weeks at a time or longer. Repeat dermal contact with weapons coated with maintenance compounds (e.g., Break-Free CLP) is also expected to occur during military operations. Proper and timely decontamination of skin (e.g., hands, forearms, face) exposed to chemicals may not be possible in the "field" or may not be recognized as a priority for preventing possible occupational injury. Occupational skin disorders are the second most common job-related disease, are underreported, and are associated with estimated total annual costs reaching $1 billion (Kabbur et al. 2001).

Break-Free CLP (Cleaning, Lubricating, Protecting) is a small-arms cleaning and maintenance compound that is commonly used by the U.S. military, police departments, and private citizens. Break-Free CLP has also been adopted as the primary weapons maintenance compound by militaries and security services of several countries including Belgium, Canada, Holland, Italy, Norway, Sweden, and Germany. Break-Free CLP is considered superior to other gun oils/lubricants because of its combined cleaning, lubricating, and metal-preserving properties which are retained under extreme environmental conditions (e.g., extreme heat/cold, humidity, mud, water, dust, etc). Break-Free CLP is a complex mixture made up of polyalphaolefin oil (65%), synthetic oils, esters and synthetic proprietary ingredients (27%), isoparaffinic hydrocarbons (5%), and dibasic ester (3%). Petroleum distillates (Meyer et al. 2000, Nessel et al. 1999) and isoparaffinic hydrocarbons (Ueno et al. 2002, Mullin et al. 1990) have been shown to have dermally irritating properties.

Recognizing that Break-Free CLP contains potential skin irritants, we carried out studies to characterize the biological effects of acute and subacute topical application Break Free CLP in CD-1 mice. In addition, this study characterized the role of inducible nitric oxide (iNOS) and its product, nitric oxide (NO), in acute dermal irritation produced by croton oil and DMBA, and the effect of Break-Free CLP exposure on this dermal irritation pathway. Increased expression of iNOS and its product, NO, are associated with inflammatory processes (Coleman 2001), and both are possibly involved in dermal irritation (Nyska et al. 2000, Vallance and Collier 1994). iNOS is expressed in keratinocytes, Langerhans cells, and macrophages (Coleman 2001, Kabbur et al. 2001, Ross et al. 1998). It is thought that iNOS expression is regulated by lipopolysaccharide or cytokine (interferon-γ, IL-1, TNF-α) produced during inflammation (Coleman 2001). During an inflammation reaction, local NO concentrations increase over several hours and can remain elevated for hours, days, or longer (Coleman 2001). It has been shown that local iNOS expression in skin is increased following topical exposure to hydrocarbons including JP-8 (Kabbir et al. 2001), m-xylene (Gunasekar et al. 2003), and 7,12-dimethylbenz[a]anthracene (Robertson 1996), indicating that NO may have a mechanistic role in hydrocarbon-induced dermal irritation. It is possible that increased local NO concentrations can be used as a marker for acute dermal irritation for other types of compounds and NO concentration levels may have utility for characterizing or discerning between responses of different degrees of severity.

Materials and Methods

Chemicals

Break-Free CLP was purchased directly from Break-Free Inc., Armor Holdings (Jacksonville, FL) in September 2001. Dermal irritation positive control compound croton oil (CO), CAS# 8001-28-3, was purchased from Sigma Chemical, Lot 89H0656. Dermal irritation positive control compound 7,12-dimethylbenz[a]anthracene (DMBA), CAS# 57-97-6, approximately 95% purity, was purchased from Sigma Chemical, Lot 31K1185. Acetone, CAS# 67-64-1, 99.5% purity, was purchased from Acros Organics N.V. and was used for diluting and dissolving CO and DMBA.

Animals and exposures

Five week old male and female mice (Crl:CD-1® BR outbred mice, Charles River Laboratories, Raleigh, NC) were single housed and acclimated for 2 weeks. Mice were randomly assigned to experimental groups of 28 mice (14 male and 14 female): On study Day 1, a 2 x 3 cm application site located directly behind the shoulder blades was clipped free of hair. Animals were then topically treated with 50 μL of deionized H_2O (dH_2O), acetone, 2.5% croton oil in acetone, or 500 μM DMBA either once or 3x week (MWF) over 2 consecutive weeks. Materials were dropped onto the skin with a pipette from 2-3 mm above the skin surface. The materials were then spread evenly over the application site with a glass rod. The application site was left uncovered (e.g., non-occluded) for the duration of the study.

Study endpoints

The chemical application site of each animal was scored by two observers for erythema and edema reactions using standard Draize test categories (Table 1). The application sites were scored at 24 hours after 1 application and 2 hours after the sixth and final chemical application on study day 12.

After 1 dermal application, 4 males and 4 females from each treatment group were anesthetized with 70% CO_2 at 24 hours following chemical application. While under anesthesia, a midline laparotomy was performed and animals were euthanized by blood collection from the hepatoportal vein using a 32 gauge needle and a 3 cc syringe. All animals were necropsied and tissues were collected for histopathological analyses. Sections were made of the following organs for histopathological examination: skin from the application site, liver, kidneys, spleen, GI tract including the colon and rectum, testes, ovaries, thymus, and axial lymph nodes. Subsections of application site skin and liver for use in mRNA and protein analysis were flash-frozen in liquid nitrogen and stored at -80°C until analyzed. Blood samples were processed and prepared for morphology and clinical chemistry analysis following standard laboratory procedures (Stiene-Martin et al. 1992).

After 6 dermal applications, the remaining 10 male and 10 female animals in each experimental group were anesthetized and euthanized on study day 12 following dermal irritation scoring. Anesthesia, euthanasia, and necropsy procedures were the same as described for animals euthanized after 1 dermal application.

Table 1 – Draize scores (Monteiro-Riviere et al. 2001)	
Erythema	Edema
0 No significant change	0 No significant change
1 Very slight erythema (barely perceptible)	1 Very slight edema (barely perceptible)
2 Slight; pale red in defined area	2 Slight edema; edges defined
3 Moderate to Severe; red in well-defined area	3 Moderate; edge raised 1 mm
4 Severe; beet red in defined area	4 Severe; raised more than 1 mm

Determination of nitric oxide production

Nitric oxide (NO) levels in skin from the application site were determined using a nitric oxide measurement kit (BioGene, US Biological, Swampscott, MA). Frozen skin sections (0.02 g) were pooled from 10 animals and were homogenized in 50 μL of RIPA buffer. Homogenate (10 μL) was then treated with nitrate reductase following the manufacturer's instructions to convert tissue nitrate (NO_3^-) to nitrite (NO_2^-). Following the addition of nitrate reductase, 2,3-diaminonapthalene (DAN) and NaOH were added to the tissue homogenate following the manufacturer's instructions for converting tissue nitrite to 1(H)-napthotriazole. It is assumed that tissue nitrate (NO_3^-) and nitrite (NO_2^-) are representative of tissue NO concentrations and that 1(H)-napthotriazole concentrations are representative of tissue NO_3^- /NO_2^- concentrations. Tissue 1(H)-napthotriazole concentrations were measured flurometrically at 375 nm with a SpectraMax GeminiXS spectrophotometer. The above procedure was repeated 3 times and tissue 1(H)-napthotriazole levels are reported as a mean concentration for the 3 replicates.

Statistical analysis

Serum chemistry concentrations and blood counts were expressed as mean ± 95% confidence interval. Statistical analyses were carried out using SYSTAT 10.2 (Systat Inc., 2002). One-way ANOVA was used to assess treatment effects at a given time. The combined effect of time and treatment was determined using factorial ANOVA. Following ANOVA, serum chemistry concentrations and blood counts were compared between treatment groups using the Tukey HSD multiple comparison test with $\alpha = 0.05$ (Ott 1988).

Results

Clinical Observations:

No deaths occurred prior to euthanasia and no clinical signs of discomfort or sickness (e.g., decreased activity, lethargy, defensive posturing, etc.) were observed in any animals topically treated once or six times with the chemicals used in our tests. Fur surrounding the application site of animals treated with Break-Free CLP appeared wet and matted with a slight copper color, which matched the appearance of the neat material. Animals from all treatment groups were observed grooming areas adjacent to the application site at 1 hour after first application. At 24 hours after first application, slight erythema (Draize score=1) was present at the application sites for 50% of the female mice treated with Break-Free CLP. Erythema was not present at the application sites of male mice treated with a single topical application of Break-Free CLP when evaluated 24 hours after treatment. Erythema was present at the application sites of both males and females treated with Croton oil (Draize score=2) or DMBA (Draize score=3) when the application sites were evaluated 24 hours after one topical treatment. No edema was observed at the application sites of any animals when evaluated 24 hours after a single topical treatment with dH_2O, acetone, Break-Free CLP, CO, or DMBA.

Repeat topical treatment with Break-Free CLP, CO, and DMBA was found to cause cumulative dermal irritation characterized by increasing amounts of erythema and edema at the site of chemical application (Table 2). Six applications of Break-Free CLP over 12 days resulted in slight erythema at the application site of 2/10 male and 3/10 female mice, respectively. Slight edema was found at the application sites of 2/3 females with erythema reactions to Break-Free CLP application. Over the course of the 6 dermal applications, it was observed that transient erythema (Draize score=1.0) occurred at the application sites of a majority of males and females within 2 hours after each Break-Free CLP application. The erythema gradually resolved to background within 8 hours of exposure with the exception of the 2/10 and 3/10 mice determined as having cumulative dermal irritation responses after 6 applications. Qualitatively, it was observed that hair growth at the application site of Break-Free CLP-treated animals was considerably less than for animals with repeat exposure to dH_2O and acetone but was significantly greater than application site hair growth for animals treated repeatedly with CO or DMBA. No lesions (e.g., open wounds or sores) were observed at the application site of animals treated with Break-Free CLP.

Moderate to severe dermal irritation reactions were observed at the application sites of animals treated repeatedly with CO. Moderate erythema and edema reactions were evident at the application sites of 10/20 animals after 6 dermal applications of CO. Animals with cumulative dermal irritation reactions also tended to have some scabbing at or adjacent to the application site.

Moderate to severe dermal irritation reactions were observed at the application sites of all 20 animals treated with 6 dermal applications of 500 μM DMBA. Animals treated with DMBA developed moderate to severe erythema and slight to moderate edema at the treatment site by the sixth topical treatment with DMBA. For most animals ($\approx$80%), the application site was bright red in color by the sixth dermal application and the skin at the application site had a raised,

wrinkled appearance. Erosion of the epidermal layer was evident for two females characterized by severe scabbing at the application site with some evidence of bleeding. No hair regrowth at the application site was evident for 3 animals (2 male, 1 female) by the sixth topical application of DMBA.

Table 2: Erythema and edema Draize scores at the conclusion of 12-day repeat dermal application toxicity testing				
	Erythema[1]		Edema[2]	
	mean	median	mean	median
Deionized H$_2$O	0.0	0.0	0.0	0.0
Break-Free CLP	0.8	0.0	0.1	0.0
Acetone	0.0	0.0	0.0	0.0
Croton oil	1.5	2.5	1.3	0.0
DMBA	3.3	4.0	2.0	3.0

Body and organ weights, serum chemistries, blood cell parameters

There were no significant differences in the body weights, relative liver weights, and relative kidney weights of animals treated once with Break-Free CLP when compared with animals treated with dH$_2$O (data not shown). There were no significant differences in body or relative organ weights for animals treated once with CO or DMBA as compared with animals treated once with acetone. No statistically significant differences occurred for serum chemistry or blood morphology parameters for animals treated once with Break-Free CLP versus those of animals treated with dH$_2$O. No statistically significant differences were found for serum chemistry or blood morphology parameters for animals treated once with CO or DMBA as compared with those for animals treated once with acetone.

Body weights and relative liver and organ weights are reported in Table 3 for animals with six topical applications over 12 days. There were no significant differences in these parameters for animals treated with Break-Free CLP versus animals treated with dH$_2$O or for animals treated with CO or DMBA as compared with animals treated with acetone.

Serum chemistry parameters are reported in Table 4 for animals with six topical applications over 12 days. Mean serum alkaline phosphatase (ALKP) activity of animals treated with Break-Free CLP was significantly lower than serum ALKP activity of animals treated with dH$_2$O. Similar results were found for animals treated with CO or DMBA versus animals treated with acetone, with the greatest decrease in activity occurring in serum from animals treated with DMBA. DMBA-treated animals also had decreased mean total protein and serum glucose concentrations and increased mean serum chloride concentrations as compared with acetone-treated animals. The overall serum chemistry profile for Break-Free CLP-treated animals was found to be significantly different as compared with the mean profile for animals treated with dH$_2$O ($p= 0.014$, Tukey multiple comparison test). Specifically, serum ALT activity for Break-Free CLP-treated animals was found to be significantly elevated ($p=0.015$) by Tukey multiple comparison test. Total protein concentrations and Na concentrations were also found to differ significantly for animals treated with Break-Free CLP as compared with dH$_2$O treated animals when analyzed by Tukey multiple comparison test. These differences were not identified when analyzed by one-way ANOVA.

Mean blood morphology parameters for animals with six topical applications over 12 days are reported in Table 5. Blood from DMBA-treated animals had significantly lower amounts of lymphocytes and higher amounts of segmented (e.g., polymorphonuclear or PMN) leukocytes as compared with animals treated with acetone.

Table 3: Body weights, relative liver and relative kidney weights at the conclusion of 12-day repeat[1] dermal application toxicity testing (95% C.I.). Results compared using One-way ANOVA.

Dose group	H$_2$O	Break-Free CLP	Acetone	Croton oil	DMBA
N	20	20	20	20	20
Final body weight	30.7 (± 2.4)	31.4 (± 2.4)	30.2 (± 2.4)	30.8 (± 2.6)	29.0 (± 2.4)
Liver weight	1.5 (± 0.2)	1.7 (± 0.2)	1.7 (± 0.2)	1.6 (± 0.2)	1.7 (± 0.2)
Relative liver weight	0.05 (± 0.00)	0.05 (± 0.00)	0.05 (± 0.00)	0.05 (± 0.00)	0.06 (± 0.00)
Kidney weight	0.5 (± 0.1)	0.5 (± 0.1)	0.5 (± 0.1)	0.5 (± 0.1)	0.5 (± 0.1)
Relative kidney weight	0.02 (± 0.00)	0.02 (± 0.00)	0.02 (± 0.00)	0.02 (± 0.00)	0.02 (± 0.00)

[1]Animals treated 3x week (MWF) for 2 weeks and euthanized 2 hours after last dermal treatment on Friday

Table 4: Blood chemistry results for animals euthanized at conclusion of 12-day repeat[1] dermal application toxicity testing (95% C.I.). Results compared using One-way ANOVA.

Chemistry	H$_2$O	Break-Free CLP	Acetone	Croton oil	DMBA
Total Protein (g/dl)	5.2 (± 0.2)	5.2 (± 0.1)	5.2 (± 0.1)	5.3 (± 0.2)	**4.6 (± 0.1)**[5]
ALKP (U/L)	110.6 (± 12.3)	**86.0 (± 11.7)**[1]	119.6 (± 12.0)	**94.3 (± 13.1)**[2]	**32.7 (± 12.0)**[3]
ALT (U/L)	20.5 (± 4.2)	24.4 (± 4.0)	20.6 (± 4.1)	24.9 (± 4.5)	23.2 (± 4.1)
GGT (U/L)	0.0	0.0	0.0	0.0	0.0
CREA (mg/dl)	0.23 (± 0.05)	0.23 (± 0.05)	0.30 (± 0.05)	0.22 (± 0.06)	0.19 (± 0.05)
GLU (mg/dl)	266.7 (± 17.1)	258.6 (± 16.3)	289.1 (± 16.7)	260.7 (± 18.2)	**193.8 (± 16.7)**[4]
Na (mmol/L)	164.4 (± 5.5)	159.6 (± 5.2)	163.8 (± 5.3)	165.0 (± 5.7)	168.5 (± 5.2)
K (mmol/L)	8.5 (± 0.7)	9.0 (± 0.7)	9.0 (± 0.7)	8.9 (± 0.7)	8.2 (± 0.7)
Cl (mmol/L)	132.7 (± 23.5)	126.6 (± 22.7)	116.2 (± 22.2)	127.1 (± 24.3)	**249.5 (± 22.2)**[6]

[1]Animals treated 3x week (MWF) for 2 weeks and euthanized 2 hours after last dermal treatment on Friday

Table 5: WBC morphology for animals euthanized at conclusion of 12-day repeat[1] dermal application toxicity testing (95% C.I.). Results compared using One-way ANOVA.

Dose group	H$_2$O	Break-Free CLP	Acetone	Croton oil	DMBA
N	20	20	20	20	20
Avg. WBC/µL	1.9 (± 0.3)	1.8 (± 0.3)	1.8 (± 0.3)	2.2 (± 0.3)	2.1 (± 0.3)
Percent segmented	11.9 (± 4.4)	17.8 (± 4.5)	12.8 (± 4.6)	19.4 (± 5.1)	**39.6 (± 4.4)**[1]
Percent banded	0.4 (± 1.1)	1.9 (± 1.1)	1.4 (± 1.0)	2.1 (± 1.0)	0.4 (± 1.0)
Percent lymphocytes	69.7 (± 5.3)	64.9 (± 5.5)	69.7 (± 5.6)	64.5 (± 6.2)	**48.9 (± 5.3)**[2]
Percent monocytes	10.5 (± 1.9)	9.8 (± 2.0)	10.8 (± 1.0)	8.6 (± 2.2)	9.3 (± 1.9)
Percent Atypical	7.2 (± 1.8)	7.4 (± 1.9)	5.9 (± 1.9)	6.9 (± 2.1)	2.4 (± 1.8)

[1]Animals treated 3x week (MWF) for 2 weeks and euthanized 2 hours after last dermal treatment on Friday

Histopathology

Histopathology of application site skin sections for Break-Free CLP-treated animals most closely paralleled those for croton oil-treated animals (Figure 1, Table 6). Foci of epithelial ulceration were observed in skin sections from 20% of Break-Free CLP animals in conjunction with markedly thickened epithelium, suggesting robust epithelial regeneration. Severe diffuse ulceration without hyperkeratosis or hyperplasia was the most common finding for skin from DMBA-treated animals (Table 6).

Examination of tissue sections from kidney, liver, gastrointestinal tract, reproductive tract, spleen, thymus, lymph node and bladder were unremarkable for animals from all treatment groups. Lesions identified in these tissues were judged to be incidental and not related to chemical exposure.

Table 6: Mean (median) lesion severity scores for animals euthanized at conclusion of 12-day repeat dermal application toxicity testing

Lesion observed	Treatment				
	dH_2O	Break-Free CLP	Acetone	Croton oil (2.5%)	DMBA (500 μM)
Ulceration[1]	0.0 (0.0)	0.3 (0.0)	0.0 (0.0)	0.9 (0.5)	4.0 (4.0)
Crust Formation[2]	0.0 (0.0)	0.2 (0.0)	0.0 (0.0)	0.4 (0.0)	-
Hyperkeratosis[3]	0.0 (0.0)	1.0 (1.0)	0.0 (0.0)	1.0 (1.0)	-
Hyperplasia[4]	0.0 (0.0)	2.0 (2.0)	0.0 (0.0)	1.7 (2.0)	0.3 (0.0)
Inflammatory infiltrates[5]	0.0 (0.0)	1.8 (2.0)	0.0 (0.0)	1.8 (2.0)	2.0 (2.0)

Lesion scoring:
[1]Ulceration: 0=normal, 1=focus limited to a region above a dermal papilla, 2= region over several hair follicles or foci above multiple papillae <25% of surface area, 3=extending over 25-50% of section, 4=>51% of section affected
[2]Crust formation: 0=normal, 1=present
[3]Hyperkeratosis: 0=normal, 1=present
[4]Hyperplasia: 0=normal, 1= 2-3X normal, 2= 4-5X normal, 3= >6X normal
[5]Inflammatory cell infiltrates: 0=normal, 1= mild increase in dermal infiltrates, 2= marked increase in dermal infiltrates

Skin NO concentrations

When measured at 24 hours after a single application, the amount of NO present in skin sections from animals treated with dH_2O did not differ significantly ($p \leq 0.05$, t-test, 2-tail) from the amount measured in skin sections from animals treated with Break-Free CLP (Fig 2).

When measured two hours after the sixth application of material, skin section NO levels for DMBA-treated animals were elevated as compared with skin NO levels from acetone-treated animals (Fig. 3), but the difference was not statistically significant ($p=0.07$, t-test, 2 tail). Skin NO levels from animals treated with CO were also elevated as compared with acetone-treated animals, but not significantly ($p=0.64$). NO levels measured in skin sections from animals treated six times with Break-Free CLP did not differ significantly from those for animals treated with dH_2O ($p=0.18$).

Discussion

Our results indicate that single 50 µL applications of Break-Free CLP (neat) do not induce skin responses indicative of dermal irritation in CD-1 mice in an open application exposure format (e.g., non-occluded). Among Break-Free CLP-treated mice, there were two distinct groups of affected animals. A majority of animals (80%) developed a localized erythema at the application site that resolved to background within 8 hours. Gross observation of the application site after six applications did not identify any morphology suggestive of a cumulative irritation response. A minority of animals (20%) treated repeatedly with Break-Free CLP developed erythema reactions that did not resolve to background between applications. Gross observation of the application site did identify morphology suggestive of cumulative dermal irritation including erythema, slight edema, scabbing, and ulceration. The dermal irritation observed in CD-1 mice as a result of repeat dermal contact with Break-Free CLP may not occur in other species; future assessments should include testing in other laboratory animal species. .

The bimodal response to repeat application of Break-Free CLP may have been the result of several factors, including individual differences in dose attributed to skin damage caused by clipping of the application site, individual variation in the amount of time that the material remained at the application site after treatment, or increased sensitivity of certain animals to Break-Free CLP as a result of genetic variation. Care was taken to ensure that the same amount of Break-Free CLP was applied to the application site at each dosing session and in the same location within the clipped area behind the shoulder blades and that animals were clipped to the same degree with minimal disturbance of the epidermis. Use of an open application system does present the possibility that the observed variation in response to Break-Free CLP exposure could be attributed to variation in residence time of the chemical at the application site. However, Break-Free CLP is an oil with relatively low viscosity and migration of the chemical from the application site to adjacent areas was limited. Differences in grooming habits between animals may have contributed to the differences in response among Break-Free CLP-treated animals. We observed that some grooming of areas adjacent to the application site occurred within hours of dermal treatment with Break-Free CLP. Individual differences in grooming could be a possible factor for the differences in response of animals to Break-Free CLP exposure. Although we applied Break-Free CLP to an area with low accessibility by the animal, grooming activities near the application site could influence the amount of material and the amount of time that the material is in contact with the application site skin. CD-1 mice are an outbreed strain, therefore individual genetic variation could account for the apparent difference in response of animals treated with Break-Free CLP.

The observed dermal irritation responses in animals treated with Break-Free CLP was not unexpected. The main ingredients of Break-Free CLP are synthetic polyalphaolefin oil, dibasic ester, and isoparaffinic hydrocarbons. Mild skin irritation was observed in rabbits and guinea pigs after single, 50 µl dermal applications of polyalphaolefin-containing hydraulic fluids (Kinkead et al. 1992, Kinkead et al. 1985, MacEwen and Vernot 1983). Dibasic ester (DBE), which contains dimethyl adipate, dimethyl glutarate, and dimethyl succinate, has been characterized as a mild to severe skin irritant in animals but is not a skin sensitizer (DuPont 1996). Repeated or prolonged dermal contact with isoparaffinic hydrocarbons causes dermatitis

(Ueno et al. 2002, Mullin et al. 1990) and may elicit contact sensitization in rare instances (Mullin et al. 1990). Dermal application of croton oil and DMBA produces dermal irritation reactions (Moon et al. 2001, Robertson et al. 1996, Ruben 1982) and repeat application of DMBA produces a contact hypersensitivity response in the skin (Klemme et al. 1987).

We did not find convincing evidence that NO production was induced in CD-1 mouse skin treated with Break-Free CLP. However, repeat dermal exposure of CD-1 mouse skin with DMBA did appear to induce NO production in skin. There was a direct correlation between increased skin NO concentrations and gross and histopathological evidence of dermal irritation in CD-1 skin treated with DMBA. The observed increase in NO concentrations is most likely due to the induction of skin NOS-2 or iNOS activity; DMBA has previously shown to cause increases in iNOS expression in dermal cells (Robertson et al. 1996). It is not clear if NO plays a pro- or anti-inflammatory role in tissue response to DMBA exposure. Depending on cell type, NO is pro-inflammatory by inducing vasodilation and the recruitment of neutrophils (Vallance and Collier 1994). Additionally, high NO concentrations promote the formation of high cellular concentrations of peroxynitrite anion ($OONO^-$) which interacts with glutathione potentially causing cellular oxidative stress (Coleman 2001). NO may also promote the down-regulation of adhesion molecules, suppression of the activation of inflammatory cells, and induces apoptosis of inflammatory cells (Coleman 2001). Also, NO may be involved in signaling for cell proliferation during tissue repair and recovery (Apte et al. 2003, Shi et al. 2001, Nyska et al. 2000).

The significant decrease in serum ALKP activity for animals treated with Break-Free CLP, CO, and DMBA suggests the liver or bone marrow may be potential organs affected in studies with longer treatment durations (e.g., 90 days). An increase in serum ALKP activity is regarded as a marker of possible liver toxicity, mainly cholestasis (Cornelius 1991), whereas low serum ALKP activities are associated with hypophosphatasia and malnutrition (Zimmerman and Henry 1969). The significant decrease in mean serum ALKP activities for Break-Free CLP and CO-treated animals may not be physiologically relevant given that normal serum ALKP levels for mice can range between 73 – 175 U/L (Cornelius 1991). No significant differences in relative liver weights occurred for animals treated with Break Free CLP, CO, or DMBA and histopathological analysis of liver sections from these animals did not find any evidence of liver toxicity. The decreases in serum ALKP activities could be the result of decreased food intake related to the gross dermal toxicity that occurred in these animals, particularly for animals treated with DMBA. The average final body weights for animals treated with Break Free CLP, CO, or DMBA were not significantly decreased as compared with controls and therefore do not support a clinical picture of malnutrition. However, a significant decrease in mean body weight as a result of decreased food intake may not be manifested in studies of short duration (e.g., <14 days).

Our findings suggest that repeat contact with Break-Free CLP could result in dermal irritation in sensitive individuals. Manufacturer's recommendations for use of personnel protective equipment (e.g., gloves) should be followed when repeat or prolonged contact with Break-Free CLP is anticipated. Use of protective gloves in a military field environment may be impractical, but persons that are expected to use Break-Free CLP should be aware that prolonged contact with the material should be avoided and washing of areas of the skin in contact with

Break-Free CLP with soap and water should be done when practical. At this time, we are not aware of any reports of dermal irritation or dermal injury among military personnel using Break-Free CLP. However, given the large number of potential dermal irritants present in a field environment (e.g., plants, hydrocarbon-based fuels, UV radiation, etc), it is possible that dermal irritation associated with any particular causative agent may not be recognized, particularly if irritation responses occur in only a small minority of users. Short of a repeat dermal contact study in volunteers, an IH survey among military personnel with frequent contact with Break-Free CLP could provide invaluable information on the actual level of risk for dermal irritation associated with frequent, repeat contact with this product. Our results suggest that tests of the dermal penetrability of Break-Free CLP should be conducted and the ability of Break-Free CLP to elicit contact sensitization should also be evaluated.

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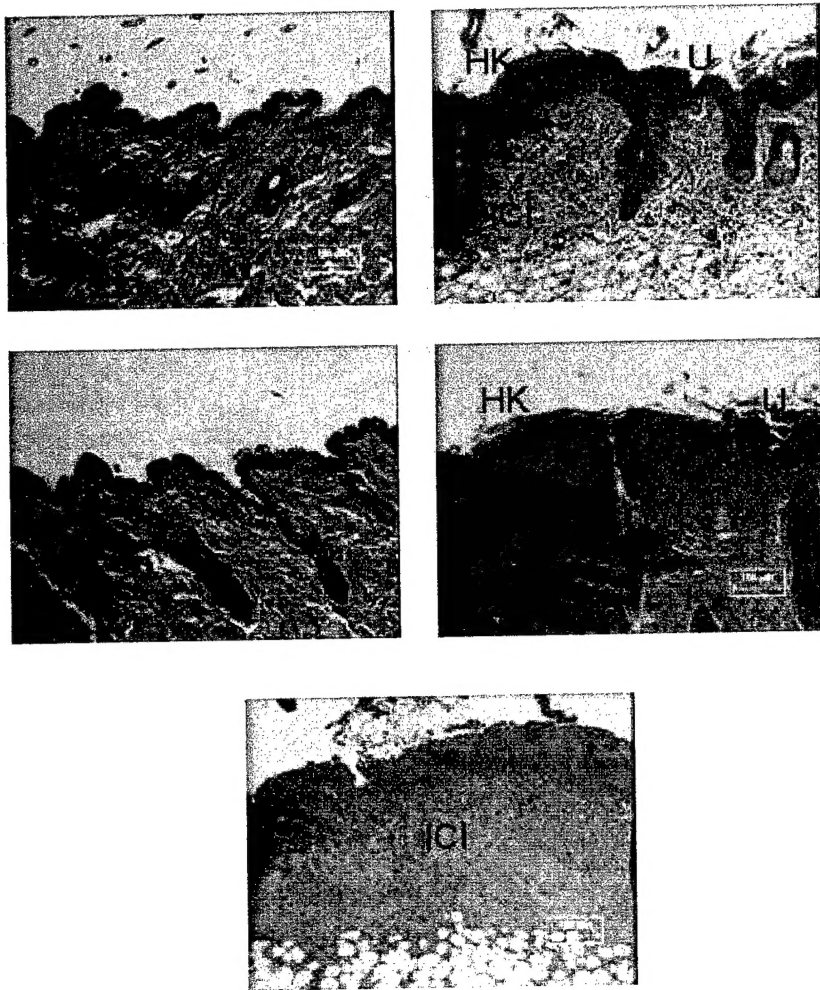
Figure 1: Application site skin sections from animals treated with dH2O (top left), BreakFree (top right), acetone (bottom left), CO (bottom right) and DMBA (bottom). Animals treated 3 x week, 2 weeks. HK=hyperkeratosis; HP=hyperplasia; ICI=inflammatory cell infiltrates; U=ulceration
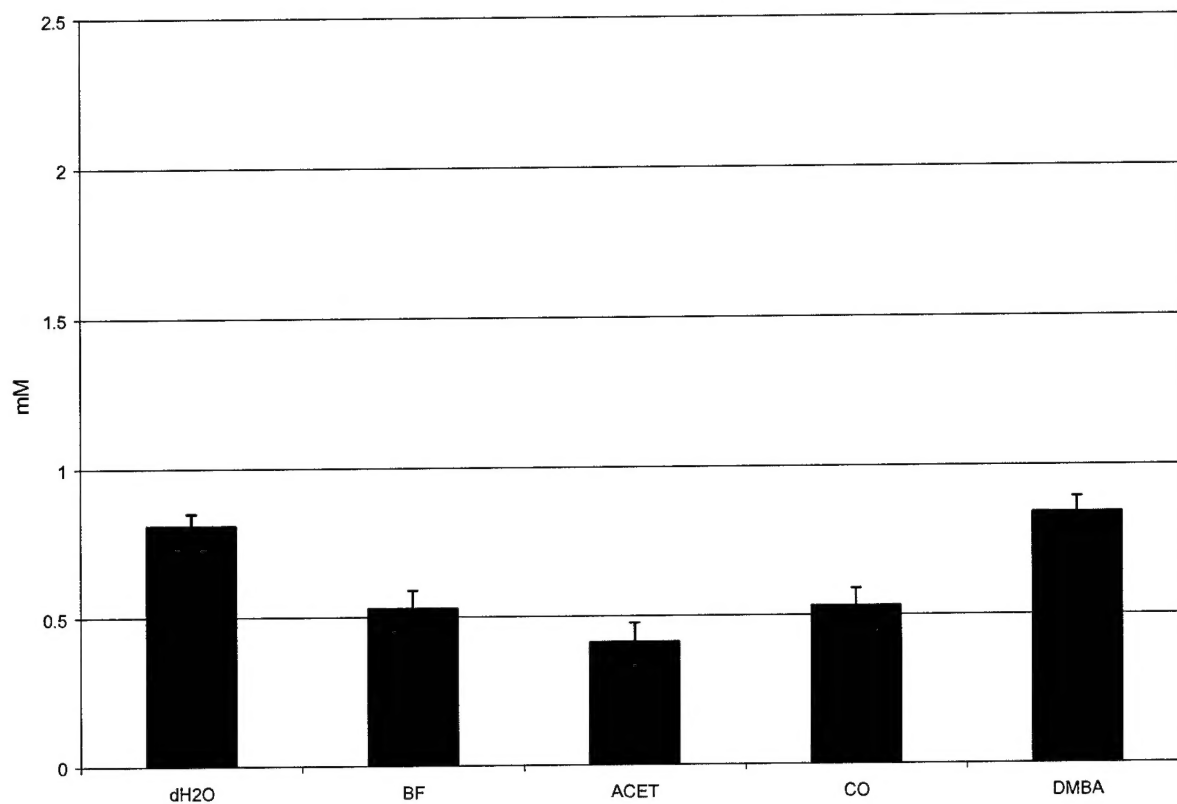
Figure 2: Skin section 1(H)-napthotriazole mean concentrations ($\pm$ 95% Confidence Level) representative of nitric oxide content 24 hours after a single application of deionized H_2O (dH2O), Break-Free CLP (BF), acetone (ACET), 2.5% croton oil in acetone (CO), and 500 μM DMBA in acetone.   * = significantly different from deionized H_2O or acetone (vehicle control) skin section concentrations
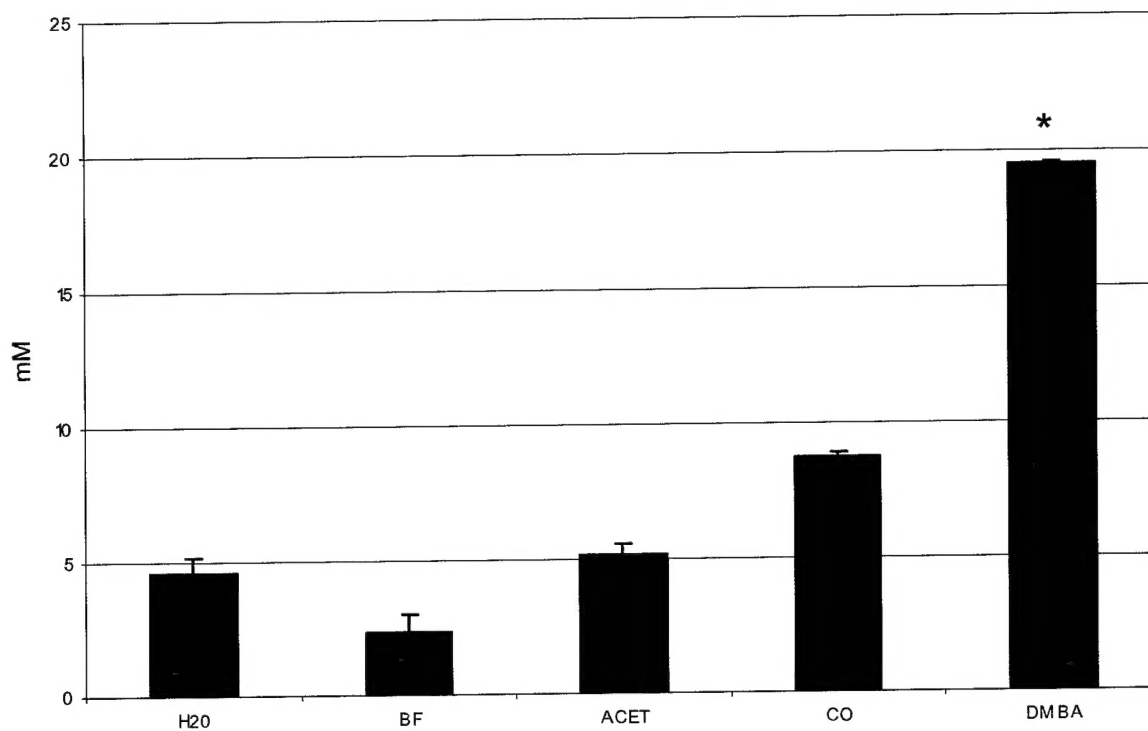
Figure 3: Skin section 1(*H*)-napthotriazole mean concentrations ($\pm$ 95% Confidence Level) representative of nitric oxide content from application site skin from animals treated 6 times (3 x week, MWF, 2 weeks) with deionized H_2O (dH2O), Break-Free CLP (BF), acetone (ACET), 2.5% croton oil in acetone (CO), and 500 μM DMBA in acetone. * = significantly different from acetone (vehicle control) skin section concentrations.

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13. SUPPLEMENTARY NOTES

14. ABSTRACT *(Maximum 200 words)*
Break-Free CLP® ("Break-Free CLP") is a commercial Cleaning, Lubricating, and Preserving compound used in both the military and civilian sectors for maintenance of small and large caliber weapons. Studies were conducted to characterize the biological effects of single or repeat dermal application of Break-Free CLP to the clipped backs of CD-1 mice. Break-Free CLP was applied neat, 50 μL/application, 3 times/week for up to 2 weeks. Foci of epithelial ulceration were observed in skin sections from 22% of Break-Free CLP animals in conjunction with markedly thickened epithelium suggesting robust epithelial regeneration. Skin histopathology of Break-Free CLP-treated animals closely matched those of mice treated repeatedly with 2% croton oil (CO) in acetone (dermal irritation positive control). Serum alkaline phosphatase (ALKP) concentrations were significantly ($p \leq 0.05$) lower for mice treated with Break-Free CLP, 2% croton oil, or 7,12-dimethylbenz(a)anthracene (DMBA) as compared with negative and vehicle control mice. Skin nitric oxide (NO) levels were not significantly elevated for mice treated with BreakFree CLP but were significantly elevated for mice treated with dermal irritation positive control compound DMBA. The cumulative skin changes in Break-Free CLP-treated animals support conducting a subchronic dermal application study. The observed decreases in serum ALKP levels suggest that future studies should include the liver and bone as possible target organs. Additionally, dermal penetration studies would provide key health risk assessment information for characterizing the potential health risks associated with chronic dermal exposure to Break-Free CLP.

15. SUBJECT TERMS
Break-Free CLP, polyalphaolefin, dermal effects, dermal toxicity, nitric oxide

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